

# Gas Chromatography–Mass Spectrometry Analysis of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in Urine and Feces

by Marlin D. Friesen,<sup>1</sup> Liliane Garren,<sup>1</sup> Jean-Claude Béréziat,<sup>1</sup> Fred Kadlubar,<sup>2</sup> and Dongxin Lin<sup>2</sup>

A method has been developed to measure levels of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) excreted in urine and feces. The method involves organic solvent extraction, derivatization to form electron-capturing *bis*-pentafluorobenzyl derivatives, and analysis by gas chromatography–negative ion chemical ionization mass spectrometry using a deuterium- labeled internal standard. The method can detect PhIP at levels of less than 1 ng/g in rat urine (5 ng/24 hr) and 5 ng/g (wet weight) in rat feces (50 ng/24 hr). Sprague-Dawley rats given a single 50  $\mu$ g dose of PhIP by gavage excreted an average of 0.6% of the dose in the urine and 25% of the dose in the feces as unchanged PhIP, in the first 4 days after treatment. To make this method applicable for the analyses of biological fluids of PhIP-exposed human subjects, it is now being improved by using immunoaffinity chromatography.

## Introduction

Epidemiological studies suggest a role for diet in the etiology of many human cancers. Among the heterocyclic amine food mutagens thus far isolated, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has been identified in fried beef and fish at levels up to 15  $\mu$ g/kg (1). The compound is mutagenic in bacteria (1) and has been shown to cause lymphomas when administered in the diet to mice (2) and colon and mammary carcinomas in rats (3). To assess possible human exposure to this compound, we have developed a gas chromatography–mass spectrometry (GC–MS) method for measuring PhIP in urine and feces. This study reports on the application and validation of this method in rats.

## Materials and Methods

### Internal Standard

A  $d_5$ -labeled PhIP internal standard was prepared by reaction of  $d_5$ -phenylalanine and creatinine, with subsequent purification by high-pressure liquid chromatography. Methane negative ion chemical ionization mass spectra of the *bis*-pentafluorobenzyl

(BPFB) derivatives of  $d_5$ -labeled and unlabeled PhIP are shown in Figure 1.

### Treatment of Rats with PhIP

Ten 7-week-old male Sprague-Dawley rats (average weight 210 g) were divided into two treatment groups and placed into individual metabolic cages. Rats were given free access to tap water, but were not fed 24 hr before treatment and for the first 48 hr after treatment. One group of five rats received a single dose, by gavage, of 50  $\mu$ g PhIP ( $\sim 250$   $\mu$ g/kg body weight) dissolved in a mixture containing 0.5 mL  $H_2O$ , 15  $\mu$ L dimethyl sulfoxide, and 0.2  $\mu$ L 0.1 N HCl to help solubility. The control group of five animals received only the solvent mixture. Urine and feces were collected at 24, 48, 72, and 96 hr and stored at  $-80^\circ\text{C}$ .

### Preparation of Urine Samples

PhIP was extracted from urine using a modification of a procedure described by Murray et al. (4). After thawing, aliquots representing 0.5% (for 24-, 48-, and 72-hr samples) or 2% (for 96-hr samples) of the 24-hr urine volumes were dissolved in 5 mL water. The pH of each sample was adjusted to 9–10 with 1 mL of 1 M  $Na_2CO_3$  and 500 pg of  $d_5$ -PhIP in 20  $\mu$ L methanol was added as internal standard.

### Preparation of Fecal Samples

Fecal samples were lyophilized, ground to a powder, and mixed to homogeneity. After weighing, 0.2% of the 24-hr and 48-hr samples and 0.8% of the 72-hr and 96-hr samples were dis-

<sup>1</sup>International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France.

<sup>2</sup>National Center for Toxicological Research, Jefferson, AR 72079.

Address reprint requests to M. D. Friesen, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France.

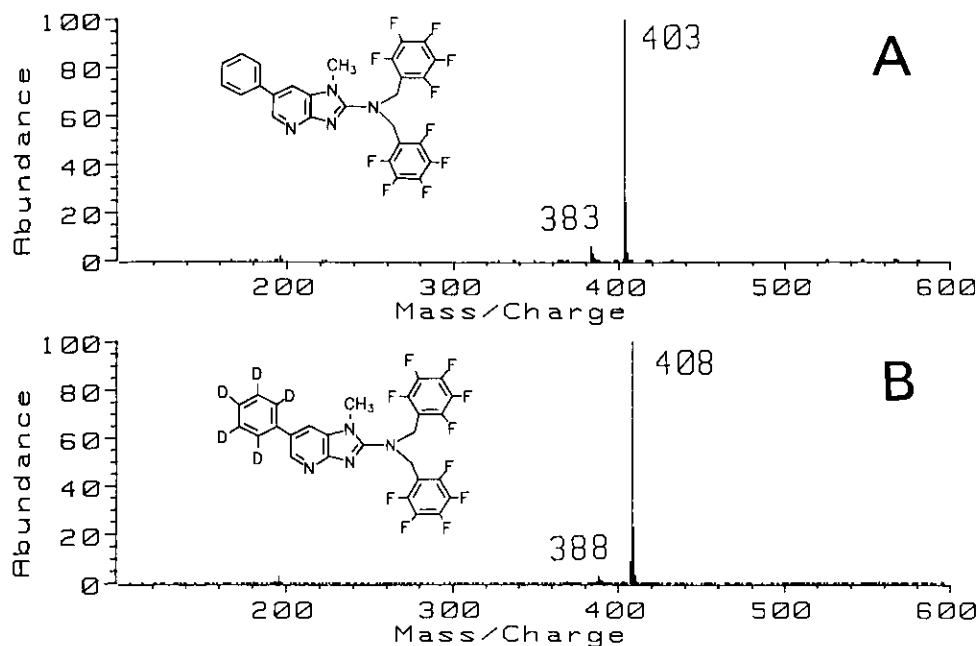


FIGURE 1. Methane negative ion chemical ionization mass spectra of (A)  $d_0$ - and (B)  $d_5$ -bis-pentafluorobenzyl-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

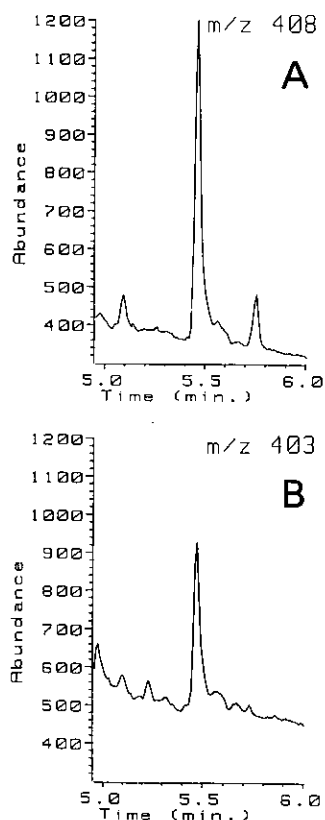


FIGURE 2. Selected ion monitoring chromatograms for (A) bis-pentafluorobenzyl-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (BPFB-PhIP)- $d_5$  internal standard, and (B) BPFB-PhIP in the urine of a rat treated with 50  $\mu$ g PhIP.

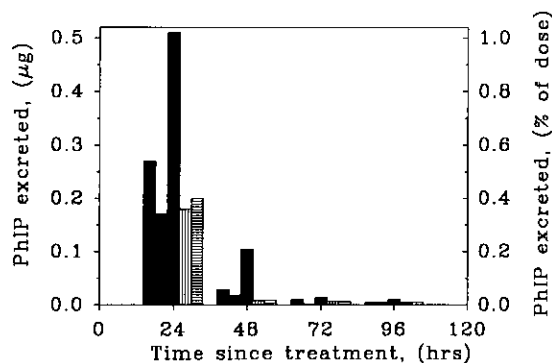


FIGURE 3. Urinary excretion of unchanged 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in five rats (represented by different bars) each treated with a single 50- $\mu$ g dose of PhIP by gavage.

solved in 5 mL water. The pH of each sample was adjusted to 9–10 with 1 mL of 1 M  $\text{Na}_2\text{CO}_3$  and 20 ng of  $d_5$ -PhIP was added in 34  $\mu$ L of methanol as internal standard.

### Extraction of PhIP from Urine and Feces

After extraction into ethyl acetate ( $2 \times 5$  mL), samples were centrifuged, and the combined organic phases were extracted with  $2 \times 5$  mL of 0.1 N HCl. PhIP was recovered from the acidic solution by addition of 1.5 mL of 1 M  $\text{Na}_2\text{CO}_3$  and extraction with ethyl acetate ( $2 \times 10$  mL). After centrifugation and decantation to remove any remaining aqueous phase, the samples were taken to dryness at ambient temperature in a Speed-Vac centrifugal evaporator.

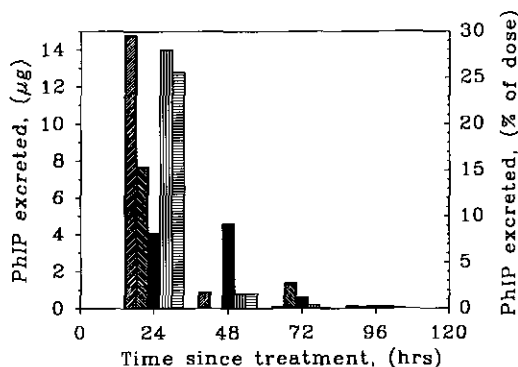


FIGURE 4. Fecal excretion of unchanged 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in five rats (represented by different bars) each treated with a single 50- $\mu$ g dose of PhIP by gavage.

## Formation of BPFB Derivatives

To obtain volatile, electron-capturing derivatives, the residues obtained after extraction from urine or feces were dissolved in a small quantity of methanol and transferred to 2-mL flame-sealable vials and taken to dryness in a centrifugal evaporator. After adding 20  $\mu$ L of a 30% (w/v) solution of pentafluorobenzyl bromide in ethyl acetate and 20  $\mu$ L of diisopropylamine, the solutions were vortex mixed. After flame-sealing, the vials were heated for 1 hr at 30°C.

After reduction to dryness under a stream of nitrogen at 30°C, the residue was dissolved in 200  $\mu$ L of 0.1 N HCl, mixed, and extracted with 2  $\times$  750  $\mu$ L hexane. The hexane was discarded. After adding 100  $\mu$ L of 1 M Na<sub>2</sub>CO<sub>3</sub> to bring the pH to 9–10, the aqueous phase was extracted with 2  $\times$  500  $\mu$ L ethyl acetate. The solution was reduced to dryness by a centrifugal evaporator in a small glass vial. The residue was dissolved in 20  $\mu$ L (for urine samples) or 200  $\mu$ L (for feces samples) of ethyl acetate for analysis by GC-MS.

## Gas Chromatography–Mass Spectrometry

GC-MS analyses were carried out on a Hewlett Packard (HP) 5890 gas chromatograph coupled through a heated interface (310°C) to a HP 5988A mass spectrometer. For urinary extracts, chromatographic separation was achieved on a HP-1 fused silica capillary column (12 m  $\times$  0.2 mm id). After splitless injection at 180°C, the column oven was heated to 290°C at 30°C/min and then at 10°C/min to 320°C. For fecal extracts, a 25 m HP-1 column was used. After splitless injection at 200°C, the column was heated at 30°C/min to 320°C, where it was held for 8 min. The mass spectrometer was operated in the negative ion chemical ionization mode with a methane source pressure of about 1 torr and source temperature of 250°C. Quantification was by selected ion monitoring of the (M-PFB)<sup>−</sup> ions of the BPFB derivatives of PhIP (*m/z* 403) and *d*<sub>5</sub>-PhIP (*m/z* 408) as shown in Figure 2 for a typical sample of rat urine.

## Results

Figure 3 shows the urinary excretion of PhIP for each of the five rats treated with a single 50- $\mu$ g oral dose of PhIP. An average of 0.6% (0.4–1.3%) of the total dose was excreted as unchanged PhIP in the first 4 days after treatment. Of this amount, an average of 84% was excreted in the first 24 hr after treatment.

Figure 4 shows the fecal excretion of PhIP for each of the five rats. An average of 25% (19–32) of the total dose was excreted as unchanged PhIP in the first 4 days after treatment. Of this amount, an average of 84% was excreted in the first 24 hr after treatment.

## Discussion

A method has been developed that can detect PhIP at levels of less than 5 ng/24 hr (1 ng/g) in rat urine and 50 ng/24 hr (5 ng/g wet weight) in rat feces. It should be possible to monitor PhIP excretion after a single dose of about 200 ng PhIP per rat. Urinary and fecal extracts were quite clean and the amounts of urine or feces extracted could have been augmented to increase sensitivity. This was not necessary in this case, however, as PhIP levels were relatively high.

Rats excreted an average of 0.6% of an oral dose of 50  $\mu$ g of PhIP in the urine and an average of 25% in the feces. These results confirm an earlier report (5) where about 2% of a 600- $\mu$ g dose in unstarved Fischer rats was excreted unchanged in the first 24 hr in the urine and 51% unchanged in the feces.

Although the results of this study are promising, the detection limit of this method may not be sufficient to allow routine quantification of PhIP at the levels expected in humans. Work is in progress to improve the clean-up step using immunoaffinity chromatography and to apply the method to matrices other than urine and feces.

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